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Adipose TriGlyceride Lipase (ATGL) and Hormone-Sensitive Lipase (HSL) protein expression is decreased in the obese insulin resistant state

5 **Short title:** ATGL and HSL in obesity

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40 **Keywords:** Adipose triglyceride lipase (ATGL); Desnutrin; Hormone-sensitive lipase (HSL); Insulin resistance; Obesity; Energy-restriction; Adipose tissue

Abbreviations: ATGL, adipose triglyceride lipase; HSL, hormone-sensitive lipase; HOMA, homeostasis model assessment; NUGENOB, nutrient-gene interactions in human obesity; IR, insulin resistant; IS, insulin sensitive.

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Abstract

Aim/hypothesis: Obesity is associated with increased triacylglycerol (TAG) storage in adipose tissue and insulin resistance. The mobilization of stored TAG is mediated by HSL and the recently discovered ATGL. The aim of the present study was to examine whether ATGL and HSL mRNA and protein expression are altered in insulin resistant conditions. In addition, we investigated whether a possible impaired expression could be reversed by a period of weight reduction.

Methods: Adipose tissue biopsies were taken from obese subjects (n=44) with a wide range of insulin resistance, before and just after a 10-week hypocaloric diet. ATGL and HSL protein, and mRNA expression was determined by Western blot and RT-qPCR, respectively. *Results:* Fasting insulin levels and the degree of insulin resistance (HOMA_{ir}) were negatively correlated with ATGL and HSL protein expression; independent of age, gender, fat cell size and body composition. Both mRNA and protein levels of ATGL and HSL were reduced in insulin resistant compared to insulin sensitive subjects (P<0.05). Weight reduction significantly decreased ATGL and HSL mRNA and protein expression. A positive correlation between the decrease in leptin and the decrease in ATGL protein level after weight reduction was observed. Finally, ATGL and HSL mRNA and protein levels seem to be highly correlated, indicating a tight coregulation and transcriptional control.

Conclusions: In obese subjects insulin resistance and hyperinsulinemia are strongly associated with ATGL and HSL mRNA and protein expression independent of fat mass. Data on weight reduction indicated that also other factors (e.g. leptin) relate to ATGL and HSL protein expression.

Introduction

Obesity is characterized by increased triacylglycerol (TAG) storage in adipose tissue and insulin resistance. The mobilization of stored TAG (lipolysis) is mediated by hormone-sensitive lipase (HSL). For more than 30 years, the paradigm has been that HSL is the rate-limiting enzyme responsible for TAG breakdown. Studies in HSL knockout mice (1-6) raised doubt on the rate-limiting role of HSL in TAG metabolism and suggested that at least one additional lipase in adipose tissue should be active that preferentially hydrolyzes the first ester bond of the TAG molecule. Recently, a new TAG lipase that belongs to a gene family characterized by the presence of a patatin-domain was identified (7-9). Zimmermann et al. termed this new non-HSL lipase: adipose triglyceride lipase (ATGL), being predominantly expressed in adipose tissue (9).

An impaired catecholamine-induced lipolysis and a reduced HSL expression in preadipocytes and differentiated adipocytes is observed in obesity (10-12). This blunted catecholamine-induced lipolysis has been proposed to be a cause of excessive accumulation of body fat. Indeed, studies in first-degree relatives of obese subjects demonstrate an impaired catecholamine-mediated lipolysis (13). Furthermore, the impaired catecholamine-induced lipolysis did not improve after weight loss, indicating that it may be an early factor in the development or maintenance of increased fat stores (14-17). A plausible other interpretation is that this reduced lipolytic response is an appropriate downregulation of lipolysis per unit fat mass to prevent an excessive fatty acid outflow from the expanded fat mass and to prevent worsening of the insulin resistant state. In line, fasting insulin concentrations have been shown to be inversely related to fatty acid efflux from adipose tissue (18).

Moreover, insulin downregulates ATGL and HSL mRNA expression in 3T3-L1 adipocytes and HSL mRNA expression is increased in adipocytes from insulin-deficient animals (19-22). In addition, ATGL is downregulated in animal models for insulin resistance (ob/ob and db/db) and HSL knockout animals show signs of impaired insulin sensitivity in adipose tissue and skeletal muscle (4, 7). Thus, there seems to be a negative relationship between insulin, ATGL and HSL expression.

The aim of the present study was to investigate whether the degree of insulin resistance and hyperinsulinemia are, independently of fat mass, related to an impaired ATGL and HSL protein expression in a group of overweight-obese subjects with a wide range of insulin resistance, selected from an existing cohort. In addition, we investigated the impact of weight loss by means of a hypocaloric diet (low-fat *vs.* medium-fat diet) on adipose tissue ATGL and HSL protein levels. To the best of our knowledge this is the first time that ATGL protein levels are measured in human adipose tissue.

Material and Methods

Subjects

This study is part of the European multicentre study NUGENOB (NUTrient-GENe Interactions in human OBesity), which is described in detail elsewhere (23-25). Only the overweight-obese subjects from the Maastricht centre participated in this part of the study. The basic selection criteria for overweight-obese subjects were age 20-50y and $BMI \geq 26 \text{ kg/m}^2$. Exclusion criteria were: weight change $> 3 \text{ kg}$ within the 3 months prior to the study start; drug treated hypertension, diabetes or hyperlipidemia; thyroid disease; surgically treated obesity; pregnancy, alcohol or drug abuse and participation in other simultaneous ongoing trials. All subjects were recruited by means of an advertisement in a local newspaper, informed in detail about the investigation and their consent was obtained before participating in the study. From the 116 participants at the Maastricht centre, a selection of 22 insulin sensitive (IS) and 22 insulin resistant (IR) subjects was made. Insulin sensitivity was assessed by the HOMA (homeostasis model assessment) index for insulin resistance ($HOMA_{ir}$) calculated from fasting glucose and insulin according to the equation of Matthews (26). The median for $HOMA_{ir}$ in the total Maastricht cohort was 2.19 (range: 0.4 - 9.9). Subjects above the 50th percentile of $HOMA_{ir}$ were assigned as IR and subjects below the 50th percentile as IS. Before entering the study, all subjects were in good health as assessed by medical history and physical examination. The Medical Ethical Review Committee of Maastricht University approved the study protocol and the clinical investigations were performed according to the Declaration of Helsinki.

Study design

A clinical investigation day took place before and just after a 10-week dietary intervention with either low-fat or medium-fat diets (see *dietary intervention*).

Subjects arrived at the clinical research centre at 8:00 a.m. after a 12 hours overnight fast and a 3-day run in period, in which they had to avoid excessive physical activity and alcohol consumption, described previously in detail (23). During this day the subjects underwent anthropometric measurements (see below) and an adipose tissue biopsy was taken (see *adipose tissue biopsy*). In addition, a venous basal blood sample was drawn for further analysis (see *biochemical analysis*).

Dietary intervention

Subjects followed one of two energy-restricted diets: a medium-fat (n=23) or a low-fat (n=21) diet. Data on the different diets and how the diet was controlled are published elsewhere (23). The target macronutrient composition of the two diets was as follows: low-fat diet, 20–25% of total energy was provided by fat; the corresponding figure for the medium-fat diet was 40–45%. Both diets derived 15% of total energy from protein and the remainder (60–65% and 40–45% for the low-fat and medium-fat diets, respectively) from carbohydrates. Both diets were designed to provide 600 kcal/day less than the individual estimated energy expenditure based on resting metabolic rate, measured using a ventilated hood system, expressed in kcal/day and multiplied by 1.3.

Anthropometric measurements

After subjects voided their bladder body weight was determined on a calibrated electronic scale, accurate to 0.1 kg. Waist and hip circumference measurements to the

nearest 1 cm were made midway between the lower rib and iliac crest with the participant standing upright. Height was measured using a wall-mounted stadiometer. BMI was calculated as body weight in kilograms divided by squared height in meters. Fat mass (FM) and fat free mass (FFM) were assessed using multifrequency bioimpedance (QuadScan 4000; Bodystat, Douglas, Isle of Man, British Isles). The percentage body fat (%BF) was calculated from total fat mass (kg) and body weight.

Adipose tissue biopsy

A subcutaneous adipose tissue biopsy was taken from the abdominal region early in the morning after an overnight fast. The second biopsy was taken in week 10 of the dietary intervention. Biopsies were performed under local anesthesia (Xylocaine® 0,5%, Lidocaine 0,5%; AstraZeneca BV, Zoetermeer, The Netherlands) on the left or right side of the abdomen about 5 cm lateral from the umbilicus using a Hepafix®-luerlock syringe (Braun Medical) and a 146x3 1/5" (2,10x80 mm) Braun-Sterican® needle. The biopsy was washed in physiological saline and stored in a sterile polypropylene tube at -80°C until further analysis.

Biochemical analysis

Plasma glucose concentrations (ABX Diagnostics, Montpellier, France) were measured on a COBAS MIRA automated spectrophotometer (Roche Diagnostica, Basal, Switzerland). Triacylglycerol (TAG) (Sigma, St Louis, USA), free fatty acids (FFA) (NEFA C kit, Wako Chemicals, Neuss, Germany) and glycerol (Boehringer Mannheim, Germany) were measured on a COBAS FARA centrifugal spectrophotometer (Roche Diagnostica, Basal, Switzerland). Standard samples with known concentrations were included in each run for quality control. Plasma insulin

and serum leptin were measured with a double antibody radioimmunoassay (Insulin RIA 100, Kabi-Pharmacia, Uppsala, Sweden; Human leptin RIA kit, Linco research, Inc, St.Charles, Missouri, USA).

5 *Fat cell volume (FCV) and fat cell weight (FCW)*

Fat cell characteristics were determined in a subset of the same cohort (n=39; 19IS/20IR; HOMA_{ir}: 1.4±0.1 vs. 4.7±0.5, P<0.01). Weight loss after diet was the same among these subjects as in the whole cohort (data not shown). Also, with respect to other metabolic parameters this subgroup was comparable to the group in which
10 ATGL and HSL protein and mRNA expression was determined (see under results). Adipose tissue was subjected to collagenase treatment, and the mean volume and weight of the isolated fat cells were determined as previously described (27).

Sample preparation

15 About 200 mg adipose tissue was ground to a fine powder under liquid nitrogen and homogenized in 200 µl of ice-cold buffer: 8M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS, Sigma C9426), 65 mM dithiotreitol (DTT, Bio-Rad 161-0611), protease inhibitor (Sigma P8340) and phosphatase inhibitor cocktail (Sigma P5726). The homogenate was vortexed for 5
20 min and centrifuged at 20000 × g for 30 minutes at 4°C. The supernatant was carefully collected and aliquots were stored at -80°C. The protein concentration was determined by Bradford based protein assay (Bio-Rad 500-0006).

Western blot analysis

Ten µg of protein were separated using 10% SDS-PAGE and then transferred to a nitocellulose membrane. An affinity purified polyclonal antibody was raised in rabbit against a 15 amino-acid peptide (amino-acids 386-400, GRHLPSRLPEQVERL) of human ATGL (Eurogentec, Seraing, Belgium). In Western blot analysis a single band at 56kDa was detected which disappeared after preincubation of the antibody with the peptide. This band corresponds to the predicted molecular mass of the human ATGL protein (9). In addition, when COS cells were transfected with cDNA coding for human ATGL, also a single band of 56kDa was observed following western blot of extracted cellular protein. HSL was detected using a rabbit polyclonal antibody, raised and purified against recombinant human HSL. The HSL antibody has been previously validated (28). The secondary antibody was a horseradish peroxidase-conjugated anti-rabbit immunoglobulin (DAKO, Glostrup, Denmark). Antigen–antibody complexes were visualized using enhanced chemiluminescence (ECL+, Amersham Biosciences, UK) and a Kodak Image Station (Kodak, Glostrup, Denmark). Quantification of antigen–antibody complexes was performed using Quantity One® 1-D analysis software (Bio-Rad). Optical density units are expressed as adjusted volume (Adj. Vol. OD, sum of pixels inside the volume boundary x area of a single pixel (in mm²) minus the background volume). Differences in loading were adjusted to β-actin protein levels and an isolated mature adipocyte lysate was included as positive control.

Adipose tissue mRNA analysis

ATGL and HSL mRNA expression was determined in a subset of adipose tissue samples of 26 subjects (13IS/13IR; 14F/12M) before and after the diet. Total RNA was

extracted from adipose tissue using the RNeasy mini kit (Qiagen, Hilden, Germany). The RNA concentration and purity were assessed spectrophotometrically. An Agilent 2100 bioanalyser (Agilent Technologies, Massy, France) was used to confirm the integrity of the RNA. From each sample, 1 µg of total RNA was reverse-transcribed to cDNA using Superscript II Reverse Transcriptase (InvitroGen, Cergy Pontoise, France) and random hexamer primers (Invitrogen). HSL and ATGL mRNAs were quantified using pre-made gene expression assays (Applied Biosystems). 18S ribosomal RNA was used as control to normalize gene expression.

10 *Statistics*

All variables were checked for normal distribution and variables with a skewed distribution were ln-transformed to satisfy conditions of normality.

First, univariate regression analysis was performed to identify variables that contribute to ATGL and HSL protein expression and to changes in protein expression

15 induced by weight loss. Subsequently, a multivariate regression analyses was performed with ATGL or HSL protein levels as dependent variables and age, gender, FM, FFM, waist circumference, circulating insulin and leptin levels as independent variables (model 1). The same model was repeated with HOMA_{ir} as independent instead of insulin (model 2). To study the impact of weight reduction, changes in

20 ATGL or HSL protein level were entered as dependent variable in the multivariate regression model with age, gender, change in fat mass, fat free mass, circulating insulin and leptin as independents. ATGL and HSL mRNA and protein levels were compared between insulin sensitive (IS) and insulin resistant (IR) subjects using Student's unpaired t-test. Anthropometric and metabolic parameters, HSL and ATGL
25 mRNA and protein levels were compared before and after the diets using Student's paired t-test. The differential effect of the diets was assessed with analysis of

covariance using diet as fixed factor. To avoid multicollinearity in the regression model independent variables with a correlation >0.8 were not simultaneously included in the model. The impact of the independent variables is described as unstandardized beta or regression coefficients. A P-value less than or equal to 0.05 was considered statistically significant. All analyses were performed using SPSS for Mac Os X version 11.0 (SPSS, Chicago, IL, USA).

Results

Characteristics of the study subjects

Anthropometric and metabolic characteristics of the study subjects before and after a 10-week hypocaloric diet are displayed in table 1. Extensive data on the effects of the hypocaloric diet in the total NUGENOB cohort were reported previously (23). The diet resulted in significant loss of body weight (before vs. after: 98.7 ± 3.2 vs. 90.0 ± 3.3 kg, $P < 0.001$), fat mass (37.4 ± 1.6 vs. 30.7 ± 1.5 kg, $P < 0.001$) and a significantly decreased BMI (34.1 ± 0.7 vs. 31.3 ± 0.7 kg/m², $P < 0.001$). In addition circulating fatty acids (506 ± 24 vs. 418 ± 22 $\mu\text{M.L}^{-1}$, $P = 0.016$), glycerol (105 ± 11 vs. 83 ± 8 $\mu\text{M.L}^{-1}$, $P = 0.003$) and leptin (24.7 ± 2.3 vs. 14.5 ± 1.5 ng/ml, $P < 0.001$) decreased. There were no significant differences in fasting glucose, insulin and HOMA_{ir}. As reported previously, the low-fat and medium-fat diet resulted in similar changes in anthropometric and metabolic parameters (23, 25).

Relationship between the degree of insulin resistance and adipose tissue ATGL and HSL protein levels

Univariate regression analysis, indicated a negative correlation between HOMA_{ir}, fasting insulin and ATGL or HSL protein levels (all $P < 0.05$), whilst age, gender, body composition (waist, FM, FFM) and levels of circulating leptin were not significantly related to ATGL or HSL protein levels (all $P > 0.10$). Multivariate regression analysis, shown in table 2, indicated the same negative correlation between HOMA_{ir} (ATGL beta-coefficient: -1.33, $P = 0.045$; HSL beta-coefficient: -0.965, $P = 0.039$; see table 2) fasting insulin (ATGL beta-coefficient: -1.41, $P = 0.048$; HSL beta-coefficient: -1.07, $P = 0.032$; see table 2) and ATGL or HSL protein levels. These data indicate that the

insulin resistant state rather than fat mass per se causes the decrease in adipose tissue ATGL and HSL protein levels.

To illustrate the impact of insulin resistance on ATGL and HSL protein levels, subjects were assigned as insulin sensitive (IS) or insulin resistant (IR) based on

5 HOMA_{ir} (see Material & Methods, *Subjects*). Anthropometric and metabolic characteristics of IS and IR subjects are displayed in table 1. Adipose tissue ATGL and HSL protein levels were found to be dramatically reduced in IR compared to IS obese subjects. ATGL protein levels were decreased by 72% in IR compared to IS obese subjects (IR vs. IS: 2.6 ± 1.3 vs. 9.3 ± 3.6 Adj.Vol.OD, $P=0.025$; see figure 1B),
10 whereas the corresponding figure for HSL was 57% (6.6 ± 2.3 vs. 15.4 ± 3.0 Adj.Vol.OD, $P=0.001$; see figure 1B). ATGL and HSL protein levels were highly correlated (beta-coefficient: 1.05, $r=0.568$, $P=0.0001$; see figure 2), indicating that ATGL and HSL protein levels might be tightly coregulated in adipose tissue of obese subjects.

15 As indicated in the methods section, FCV and FCW were determined in a subset of insulin sensitive ($n=19$) and insulin resistant ($n=20$) subjects from the same cohort with similar characteristics with respect to HOMA_{ir} (1.5 ± 0.1 vs. 4.7 ± 0.5 ; $P<0.01$), body fat % (42.5 ± 1.1 vs. 42.8 ± 2.0 ; $P=0.742$) and waist (108 ± 2 vs. 114 ± 2 ; $P=0.031$) and similar diet-induced changes. Fat cell volume (FCV) and fat cell weight (FCW)
20 were significantly higher (12%; $P=0.039$) in IR compared with IS subjects and decreased significantly after weight loss ($P<0.01$), see table 1. When ATGL and HSL protein expression was corrected for mean FCV or FCW the difference between groups remained significant (for ATGL, IR vs. IS: 2.6 ± 0.6 vs. 3.9 ± 0.5 , $P<0.01$; for HSL, IR vs. IS: 9.2 ± 1.5 vs. 13.4 ± 1.6 , $P<0.01$).

ATGL and HSL mRNA expression in insulin sensitive versus insulin resistant subjects

Additionally, ATGL and HSL mRNA expression was determined in a subset of adipose tissue samples of 26 subjects. In agreement with the protein expression data, ATGL and HSL mRNA expression were significantly lower in IR (n=13) compared to IS (n=13) obese subjects (P=0.006 and P=0.057, respectively; see figure 1A). Also, a positive correlation was found for ATGL and HSL mRNA expression (beta-coefficient: 0.531, $r = 0.253$, P=0.005), suggesting that the two enzymes belong to a common regulatory network with tight transcriptional control.

10 *Effect of weight reduction on adipose tissue ATGL and HSL protein levels*

A 10-week hypocaloric diet resulted in a decreased adipose tissue ATGL (before vs. after 5.7 ± 1.8 vs. 1.4 ± 0.4 Adj.Vol.OD, P=0.04; see figure 3B) and HSL (before vs. after: 10.8 ± 1.9 vs. 5.9 ± 1.3 Adj.Vol.OD, P=0.023; see figure 3B) protein level. When ATGL and HSL protein expression was corrected for mean FCV or FCW the difference remained significant (for ATGL, before vs. after: 3.2 ± 0.5 vs. 2.1 ± 0.4 , P=0.02; for HSL: before vs. after: 11.1 ± 1.8 vs. 7.5 ± 1.4 , P<0.01). Low-fat and medium-fat diets resulted in similar changes in ATGL and HSL protein levels. To find out the effect of changes in anthropometric and metabolic parameters on ATGL and HSL protein levels univariate and multivariate regression analysis was applied (see table 3). Univariate regression analysis, indicated a positive correlation between the decrease in leptin and the decrease in ATGL protein level after weight reduction (P<0.05) whilst age, gender, changes in body composition (FM, FFM) and insulin were not significantly related to changes in ATGL protein level (all P>0.10). Multivariate regression analysis (see table 3) indicated the same positive correlation between the decrease in leptin and the decrease in ATGL protein level after weight

reduction (beta-coefficient: 6.05^E-02 , $P=0.023$; see table 3). In addition, univariate regression analysis indicated a positive correlation between the decrease in FFM and the decrease in HSL protein level after weight reduction ($P<0.05$) whilst age, gender, changes in fat mass, circulating levels of insulin and leptin were not significantly
5 related to changes in HSL protein level (all $P>0.10$). Multivariate regression analysis (see table 3) indicated the same positive correlation between the decrease in FFM and decrease in HSL protein level after weight reduction (beta-coefficient: 0.67, $P=0.020$; see table 3).

Finally, the correlation between ATGL and HSL protein levels found during habitual
10 dietary conditions was also observed after a 10-week hypocaloric diet (beta-coefficient: 0.99, $r=0.484$, $P<0.0001$). Also, changes in ATGL and HSL induced by weight loss highly correlated to each other (beta-coefficient: 1.01, $r=0.503$, $P=0.004$; see figure 4). This coordinated variation in ATGL and HSL expression during various dietary conditions suggests that the two enzymes share a common regulatory network.

ATGL and HSL mRNA expression after the diet

In line with the protein expression data a 10-week hypocaloric diet significantly lowered adipose tissue ATGL ($P=0.001$) and HSL ($P=0.007$) mRNA expression, see figure 3A. Low-fat and medium-fat diets resulted in similar changes in ATGL and
20 HSL mRNA expression. A strong positive correlation was found between ATGL and HSL mRNA expression after the diet (beta-coefficient: 0.761, $r=0.578$, $P<0.0001$). The changes in ATGL and HSL mRNA expression induced by weight loss tended to correlate positively ($P=0.135$).

Discussion

Our study, for the first time, examined the relationship between adipose tissue ATGL and HSL mRNA and protein expression, and whole-body insulin sensitivity in a group of obese subjects. The major finding of the present study is that a reduced ATGL and HSL mRNA and protein expression is associated with insulin resistance independent of fat mass. Weight reduction decreased, rather than increased ATGL and HSL mRNA and protein expression. When ATGL and HSL protein expression was corrected for mean FCV or FCW the differences between groups remained significant. In addition, ATGL and HSL mRNA and protein expression seem to be tightly coregulated in adipose tissue, suggesting that they belong to a common regulatory network.

Our data indicate that the degree of insulin resistance and hyperinsulinemia in obesity rather than the increase in fat mass and body fat distribution per se is associated with a reduced ATGL and HSL protein and mRNA level. Since we only studied expression in abdominal subcutaneous fat we cannot rule out depot-specific differences in ATGL and HSL expression. For instance, it has been shown that HSL mRNA expression is significantly different in subcutaneous and visceral adipose tissue, a finding that could not be confirmed for ATGL mRNA expression (29). There is accumulating evidence from *in vitro* and animals studies that insulin reduces HSL and ATGL expression. It is documented that insulin downregulates ATGL and HSL mRNA levels in 3T3-L1 adipocytes in a dose dependent manner (19, 21, 22). More interestingly, ATGL is downregulated in a mouse model for insulin resistance by 50% (7). In accordance, our data indicate a 72% reduction in adipose tissue ATGL protein levels of insulin resistant compared to insulin sensitive obese subjects. Moreover, HSL mRNA levels

are increased in adipocytes from insulin-deficient streptozotocin-treated rats as compared to controls suggesting a negative effect of insulin on HSL expression (20).

Weight reduction decreased, rather than increased ATGL and HSL protein and mRNA expression levels with no effect of diet composition and independent of

5 changes in fat mass. This seems consistent with Viguerie et al. reporting a similar decrease in HSL mRNA for the low-fat and medium-fat diet (25). This

downregulation of key enzymes for triglyceride breakdown and the increase in lipoprotein lipase mRNA level after weight loss (30), potentially enhances lipid storage and making further weight loss more difficult. In contrast, Mairal et al.

10 showed that adipose tissue ATGL mRNA expression was unchanged and HSL mRNA expression increased after long-term weight reduction in obese subjects (29). It should be mentioned that in this study the second biopsy was taken 2-4 years after surgery. A

factor explaining the inconsistent findings may be that different conditions are compared. In the present study subjects were investigated while still on the energy-

15 restricted diet (second biopsy taken just at the end of the diet). The negative energy balance produced by the energy-restricted diet is known to profoundly modify

adipocyte metabolism, particular the lipolytic pathway, making it impossible to differentiate between the chronic effect of weight reduction per se and the acute effect of energy restriction. Interestingly, the decrease in leptin correlated positively with the

20 decrease in ATGL expression after energy restriction, independent of changes in fat mass, fat free mass and circulating insulin levels. Flier et al. advocate that this

decrease in leptin concentration serves as an important signal from fat to the brain that the body is starving (31). In addition, it has been proposed that an important function of leptin is to confine storage of triglycerides to adipocytes (i.e. to affect adipose

tissue lipolysis) and to prevent triglyceride storage in non-adipocytes (e.g. myocytes), protecting them from lipotoxicity (32).

In obese subjects we observed no strict relationship between fat mass and ATGL or HSL expression, and fat cell size per se was not important for our findings. When the

5 obese state has already developed insulin resistance and hyperinsulinemia seem to be the major determinants for ATGL and HSL protein expression. This seems in line with the observation of a negative correlation between fasting insulin and *in vivo* fatty acid outflow per unit of adipose tissue in insulin resistant conditions (18), suggesting that a reduced expression of ATGL and HSL may be a secondary phenomenon to

10 insulin resistance. It can be speculated that hyperinsulinemia may downregulate adipose tissue lipolysis and thereby prevent worsening of the insulin resistant state (33, 34). In the present study weight loss had no significant effect on insulin

sensitivity. To fully elucidate the effect of insulin resistance on ATGL and HSL expression an intervention should be performed which significantly improves insulin

15 sensitivity (e.g. exercise training or treatment with a PPAR- γ agonist). It has been shown that ATGL is subject to transcriptional control by PPAR- γ mediated signals (22). In addition, Festuccia et al. recently showed that treatment of mice with the PPAR- γ agonist rosiglitazone significantly increased ATGL and HSL mRNA expression (35), indicating that an improved insulin sensitivity

20 increases adipose tissue ATGL and HSL expression. Finally, we cannot rule out that a decreased ATGL and HSL expression is a primary defect in obesity. Interestingly, ATGL-deficient mice have an increased fat storage in adipose and non-adipose tissues (36). Further, studies in first-degree relatives of obese subjects have demonstrated an impaired lipolytic function of adipocytes, suggesting that also primary adipocyte

25 lipolysis defects are present in obesity (13). Expression of HSL is markedly decreased

in subcutaneous adipocytes and differentiated preadipocytes from obese subjects, suggesting a decreased HSL expression to be a primary defect in obesity {Large, 1999 #244; Lofgren, 2002 #263; Viguerie, 2005 #259; Reynisdottir, 1994 #234}. Also several studies suggest that genetic variation in the *HSL* and *ATGL* gene are associated with obesity and type 2 diabetes mellitus (37, 38). Further research is needed to elucidate the exact order of events.

The coregulation between ATGL and HSL protein levels or mRNA expression (39) during different dietary conditions suggests that the two enzymes belong to a common regulatory network with tight transcriptional control. A recent study indicated that HSL is the major lipase catalyzing the rate-limiting step in stimulated lipolysis, whereas ATGL participates in basal lipolysis (39). Insufficient time has passed since the discovery of ATGL to understand the nature of its regulation. However from the limited data available, it appears that in comparison to HSL, ATGL is not a direct target for protein kinase A (PKA)-mediated phosphorylation and is localized on the lipid droplet in the basal and hormone-stimulated state of the cell (9). These observations suggest that ATGL is not activated by phosphorylation and translocation to the lipid droplet as demonstrated for HSL. Instead, an activator protein regulates ATGL activity: CGI-58 (comparative gene identification 58) (40) (41). It will be important to establish whether the decreased HSL and ATGL protein and mRNA expression observed in insulin resistant subjects is also accompanied by a decreased activity of both enzymes.

In conclusion, ATGL and HSL expression are decreased in the obese insulin resistant state. When the obese state has already developed insulin resistance or hyperinsulinemia seem to be the major determinant of ATGL and HSL protein expression independent of fat mass. On the other hand, there are also indications that

a reduced ATGL and HSL protein expression is a primary defect in obesity. Weight reduction decreased ATGL and HSL expression, independent of circulating insulin and FM, indicating that also other factors (e.g. leptin) relate to ATGL and HSL protein expression in obese subjects.

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